Disruption of natural killer cell homing as a biomarker in persons aging with or without HIV

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Abstract

Natural killer (NK) cells are critical modulators of HIV transmission and disease. While recent evidence suggests a loss of NK cell cytotoxicity during aging, a compound analysis of NK cell biology and aging in persons with HIV (PWH) is lacking. We set out to perform one of the first large comprehensive analyses of people aging with and without HIV to determine NK phenotypic changes during aging and how these changes are modulated while aging with HIV. Utilizing high-dimensional polychromatic flow cytometry we analyzed 30 immune-related proteins spanning broad functions such as trafficking, activation/inhibition, NK specific receptors, and memory/checkpoint receptors on peripheral NK cells from health donors, PWH with viral suppression, and viremic PWH. NK cell phenotypes are dynamic across the age span but are significantly altered in HIV and ART and with co-factors such as CMV. Specifically, NK cells in healthy aging show increasing levels of α4β7 and decreasing CCR7 expression during aging, a phenomenon nearly perfectly reversed in PWH. These HIV-associated trafficking changes could be in part due to NK cell recruitment to HIV reservoir formation in lymphoid tissue or failed mucosal signaling in the HIV-infected gut, but regardless appear to be tight biomarkers of age-related NK cell changes.

58 **Introduction**

59 Advances in combination antiretroviral drug therapy (cART) and early intervention led to
60 HIV becoming a chronic illness and an increase in life expectancy of people with HIV
61 (PWH)(1-3). Consequently, the population of PWH are aging, with those over the age of
62 50 accounting for over 20% of PWH worldwide(4) and 51% in the United States(5).
63 However, the necessity of long-term cART poses a potentially serious problem as PWH
64 age as its impact on the immune system is still not completely understood(6). Chronic
65 inflammation is suggested to be a leading factor of morbidity during aging(7) and PWH
66 exhibit this chronic inflammation despite long-term viral suppression(7). Furthermore,
67 aging PWH are at higher risk of age-related comorbidities, such as cardiovascular
68 disease, and polypharmacy is common, which can lead to potential drug-drug
69 interactions(8). The progression and burden of age-related comorbid conditions and
70 multimorbidity in people aging with HIV (PAWH) differs proportionally in several ways to
71 the general uninfected population, however the mechanisms and impact of viral directed
72 immune responses is still not completely understood.

73 Natural killer (NK) cells, potent innate immune cells important in viral and tumor
74 surveillance and immunoregulation, have been shown to play a critical role in HIV(9).
75 Specific killer immunoglobulin-like receptors (KIRs) and human leukocyte antigen (HLA)
76 combinations have been shown to be highly effective at control and protection from HIV
77 infection(10,11). Additionally, it has been shown that high NK cell functional capacity is
78 closely associated with inhibited HIV transmission(12,13). Interestingly, in non-human
79 primate models of simian Immunodeficiency virus (SIV), NK cells are also shown to be
80 highly plastic and undergo large shifts in trafficking to lymph nodes and/or gut mucosal

81 sites(14-16). However, NK cells have also been shown to become exhausted during
82 chronic HIV infection with typical signs being increased frequency of $CD56^{dim}$ $CD16^{+}$ NK
83 cells but a decrease in functional responses(17) and a loss of Siglec-7 expression(18).
84 Furthermore, aging has also been characterized by chronic low-grade inflammation,
85 alterations and dysfunction in adaptive immune responses, and changes in innate
86 immune cells(19,20). NK cells in aging show similar dysfunctions that are seen in chronic
87 HIV infection, namely an increase in $CD56^{dim}$ $CD16^{+}$ proportions but lower functional
88 capacity(21-23) which results in an increased risk of infection(24). In addition to an
89 increase in raw numbers of NK cells with age they also have reduced proliferative
90 potential(21), decreased surface expression of the activating receptors NKp30 and
91 NKp46(21), modulated KIR expression(21), loss of Siglec-7 and Siglec-9 expression(25),
92 an accumulation of senescent cells that may be a result of age-related decline in NK
93 cytotoxicity(26-28), and age-related trafficking changes that are directly responsible for
94 increased susceptibility to certain pathogens(29).

95 To understand mechanisms underlying the interactions between NK cells, aging
96 and HIV control, we examined NK cell phenotypic changes in PWH either on effective
97 ART or untreated across a broad age span. We developed a high-dimensional flow
98 cytometry comprehensive panel to measure 30 immune-parameters consisting of
99 trafficking markers, NK cell receptors, activating/inhibitory receptors, and senescent cell
100 markers. By defining HIV and aging specific NK cell perturbations, this will allow for the
101 development of novel approaches to limit or reverse innate immune dysfunction, alter
102 trajectories of co-morbidities and improve clinical outcomes in PAWH.
103

Results

Study design and cohort demographics

For our analysis we utilized a cohort of 135 samples collected by the Hawai'i Aging with HIV-1 Cohort (HAHC) at University of Hawai'i, comprised of healthy donors (HD; n = 49), PWH on treatment (cART; n = 61), and PWH off treatment (Viremic PWH; n = 25). The three groups had similar age ranges with HD having a median age of 48.01 years (32.48 – 73.48), cART having a median age of 52.99 years (26.67 – 73.34), and Viremic PWH having a median age of 42.29 years (22.28 – 78.07) (Supplemental Table 1). Sex proportion, duration of known infection, viral load, and other clinical information can be found in Supplemental Table 1. High-dimensional polychromatic flow cytometry was utilized for this study and used to quantify surface protein expression of a broad array of receptors. The panel designed covered proteins that broadly fell into (i) trafficking receptors, (ii) activation/inhibition receptors, (iii) adaptive/memory markers, and (iv) immune exhaustion markers (Supplemental Table 2).

NK cells in aging healthy donors show paucity of receptor repertoires

To determine the impact of HIV on NK cells during aging, we first examined HIV uninfected donors (HD) in two age stratifications (under the age of 45; and over the age of 50) to establish a baseline “aged phenotype” of NK cells in the absence of known HIV infection. The median age of HD was 48.01 years old with a range of 32.48 – 73.48 years old. We focused our analyses on the dominant blood phenotype of cytotoxic $CD56^{dim}$ $CD16^{+}$ NK cells. Representative gating strategies for each group are shown in Supplemental Figure 1. Generalized Linear Modeling (GLM) with bootstrap resampling

127 was employed to examine the log-odds of protein expression being a predictor of age
128 group (Figure 1A). GLM analysis results indicated that CD127 (IL-7Rα) increase with
129 aging is a significant predictor of aging in HD (p = 0.021; Figure 1A). In contrast, lower
130 CD8α (p = 0.021) and CCR7 (p = 0.042) expression are significant predictors of younger
131 people (Figure 1A) while KIR3DL1S1 showed a trend towards younger individuals (p =
132 0.063; Figure 1A). Interestingly, CCR7 was a significant predictor (p = 0.042) and
133 negatively correlated with age (R = -0.5219, p = 0.0005; Supplemental Figure 2D), while
134 the gut-homing marker α4β7 directly correlated with age (R = 0.4057, p = 0.0085;
135 Supplemental Figure 2D) but was not a predictor of age in the GLM analysis (p = 0.978;
136 Figure 1A). This surprising finding suggested that changes in NK cell homing repertoires
137 may be associated with age. We also sought to utilize dimensionality reduction methods
138 to examine the high-dimensional data on a 2-dimensional projection to interrogate
139 whether the two age groups cluster independently. We first used Multi-Dimensional
140 Scaling (MDS) and found that both age groups largely overlapped, highlighting that NK
141 cells in aging remain phenotypically consistent during aging (Figure 1B). Barnes-Hut
142 implementation of t-Distributed Stochastic Neighbor Embedding (bh-SNE) analysis
143 further indicated that the two age groups largely overlap with minimal distinct clusters
144 (Figure 1C). Relative expression overlaying of bh-SNE maps shows that the predictors
145 identified previously, CD127, CD8α, and CCR7, largely do not show distinct clustering
146 except for CCR7 which show a clearly distinct NK subset expressing high levels (Figure
147 1C; CD127 Top Right, CD8α Bottom Left, and CCR7 Bottom Right). We finally examined
148 the significant predictors from the GLM analysis using violin plots (Figure 1D) to visualize
149 expression levels between the two age groups and find that expression levels are

150 consistent with their predictions with CD127 showing higher expression in aging while
151 CD8α, CCR7, and KIR3DL1S1 showing decreased expression in the younger cohort.
152 Importantly, each of these clusters and groupings supported the GLM and standard
153 correlative analyses indicating roles for CD8α , CD127, and changes in homing for NK
154 cells during aging.
155

156 **NK cell aging phenotypes in PWH diverge in people over 50 compared to uninfected**
157 **controls**

158 Next, we sought to determine the impact of HIV infection on the NK cell aging phenotype
159 seen in HD. We examined two groups of PWH: those that were either on cART or not on
160 treatment (Viremic) at the time of sampling (Supplemental Table 1). Using the same GLM
161 with bootstrap resampling as before, we first examined PWH compared to HD for those
162 under the age of 45. Surprisingly, there were few significant predictors of HIV status in
163 this age group (Figure 2), with only PD-1 and HLA-DR being significant when HD were
164 compared to cART (p $p = 0.021$ and $p = 0.021$, respectively; Figure 2A). When comparing
165 HD and Viremic under the age of 45, NKG2C and α4β7 were potential predictors of HIV
166 ($p = 0.084$ and $p = 0.084$, respectively; Figure 2B) status while PD-1 was a predictor of
167 the HD group ($p = 0.042$; Figure 2B). Interestingly, α4β7 had a significant negative
168 correlation with age in both the cART (Supplemental Figure 3D) and Viremic groups
169 (Supplemental Figure 4D), which is inverse to the correlation seen in HD aging
170 (Supplemental Figure 2D), again suggesting that aging with HIV causes a shift in NK cell
171 trafficking. In contrast to the under 45 group, many proteins emerged as significant
172 predictors of both HIV groups for the over 50 group. For PWH on cART over the age of

173 50, CD2, CCR7, and α4β7 emerged as significant predictors compared to HD (p = 0.014,
174 p = 0.014, and p = 0.031, respectively; Figure 2C). These predictors were consistent in
175 the Viremic group (p = 0.021, p = 0.021, p = 0.021, respectively; Figure 2D), in addition
176 the proteins CCR5, CD85j, CCR7, and HLA-DR were also potential predictors of Viremic
177 PWH compared to HD (p = 0.0945, p = 0.021, p = 0.021, and p = 0.084, respectively;
178 Figure 2D). All together these results again strongly indicate NK cell trafficking is
179 modulated in HIV and aging, as shown by a decreased expression of the gut-homing
180 marker α4β7 and increased expression of the lymph node homing marker CCR7. Broadly
181 this change could be considered a reversal of the expected healthy aging homing
182 repertoire of NK cells.
183

184 **HIV viral load and duration of known infection predicts NK cell repertoires in**
185 **persons aging with HIV**

186 NK cell changes in functional capacity, phenotype, exhaustion, and trafficking have been
187 previously shown to correlate with viral load(30-34). Due to these previously described
188 correlations, we next aimed to evaluate potential correlates among HIV viral load and
189 observed NK phenotypes in the Viremic group, independent of age. Interestingly, viral
190 load did not correlate with NK cell frequency (Figure 3A), activation/checkpoint markers
191 (Figure 3B), nor NK cell receptors (Figure 3C). Surprisingly, only CCR7 significantly
192 correlated with viral load (R = 0.4804, p = 0.0275; Figure 3D). None of the other trafficking
193 markers showed significant correlations; however, α4β7 did show a positive trend with
194 viral load (R = 0.3758, p = 0.0932; Figure 3D). These analyses reenforce the fact that

195 even independent of aging, HIV infection and ongoing virus replication influences NK cell
196 homing and potential tissue localization.

197 Given the duration of HIV infection may play a role in altered NK cell phenotype in
198 PWH, we performed correlation analysis between NK cell markers and duration of known
199 infection for PWH with or without cART (Figure 4). Unexpectedly, a significant positive
200 correlation with duration of known infection and frequency of NK cells in PWH on cART
201 was observed (Figure 4A). Interestingly, only 2B4 showed a positive trend with duration
202 of infection among Viremic PWH (R = 0.3918, p = 0.0790; Figure 4B). NK specific
203 receptors did not correlate with duration of infection, except for NKp46 in the cART group
204 showing a weak negative correlation (Figure 4C), and correlations between duration of
205 known infection and NK trafficking were not observed (Figure 4D). Collectively, these data
206 suggest duration of infection could influence the NK cell repertoire, but rather age and
207 viremia are predictors of the HIV-associated trafficking change.

208

209 **Elevated CMV IgG titers is linked to higher expression of several NK cell aging**
210 **phenotypes in those over 50**

211 NK cells are known to undergo phenotypic and functional changes following CMV
212 infection, mainly through the expansion of an adaptive NK pool that are $CD57^+$ $NKG2C^+$
213 (35-37). To determine if age-related changes observed are due to CMV infection we
214 evaluated plasma anti-CMV IgG antibody titers in all cohort groups. Consistent with
215 known data on CMV and HIV co-infections(38), CMV antibody titers were elevated in
216 PWH compared to HD (Supplemental Table 1). The CMV+ HD over 50 years of age had
217 higher proportions of NK cells expressing CD57, NKG2C, CD127, PD-1, and CD85j

218 (Figure 5A), which could represent an expanded adaptive NK subset previously described
219 by our group and others(35-37,39). Interestingly, we did not see a significant impact of
220 CMV on α4β7 or CCR7 in HD (Figure 5B-5D). However, increased CCR5 expression is
221 observed with CMV titers in both HD age groups (Figure 5E) and positively correlates
222 with age (Supplemental Figure 5D). Overall, these data suggest that CMV is a modulator
223 of adaptive NK cells and other NK cell receptors, but changes in PWH are largely
224 dominated by age and HIV status (Supplemental Figure 8). Specifically, HIV and age
225 drive the CCR7/α4β7 trafficking change independent of CMV (Supplemental Figures 3,4
226 6-8). Even with the limited data within range we do see a significant negative correlation
227 in the cART group between CXCR6 expression and CMV IgG titer (R = -0.3721, p =
228 0.0072; Supplemental Figure 6D). However, we do not see the same correlation in the
229 Viremic group for CXCR6 (R = 0.3404, p = 0.1311; Supplemental Figure 7D). Chord plots
230 are consistent with these findings as well, showing that it appears that HIV is the main
231 driver of NK phenotypic changes in both the cART and Viremic groups (Supplemental
232 Figure 8).
233

234 **Discussion**

235 Delineating meaningful changes in NK cell profiles during aging and in PWH has
236 remained poorly understood, limiting a complete understanding of the cellular changes
237 provoked following infection that may result in subsequent secondary disease states. To
238 help minimize this gap we used 28-color polychromatic cytometry to robustly characterize
239 cell surface changes in the NK cell repertoire of healthy persons and persons aging with
240 HIV on and off cART. Indeed, we found one of the tightest predictors of how the global
241 NK cell repertoire changes during healthy aging can be predicted solely by increasing
242 α4β7 expression. Furthermore, we find a total reversal of this phenomenon in PWH.

243 Aging has been found to increase the $CD56^{dim}$ $CD16^{+}$ of NK cells while decreasing
244 functional capacity and even further contributing to systemic inflammation(24). Our data
245 demonstrated a well conserved aging NK phenotypic profile consisting of NK cell specific,
246 activation and altered checkpoint specific receptors. Interestingly, a highly distinctive
247 trafficking pattern was identified with NK cells in aging consisting of decreased lymph
248 node homing via downregulation of CCR7, in conjunction with increased gut mucosae
249 homing via upregulated α4β7. Previous reports have identified age-associated
250 dysfunction in the gut integrity resulting in microbial translocation and macrophage
251 dysfunction(40). Our data could suggest that increased recruitment of NK cells to the gut
252 mucosae could be one mechanism for maintaining gut homeostasis during aging. Further
253 investigation into the cytolytic capabilities of trafficked NK cells to the gut mucosae can
254 provide more robust insights into the identified trafficking repertoire.

255 Introduction of cART therapy has dramatically lengthened the lifespan of PWH,
256 resulting in a novel cohort crucial to understanding HIV pathogenesis. One of the most

257 interesting findings in this study was the modulation of NK cell trafficking pattern with HIV
258 infection. Regardless of cART treatment, NK cells from PWH showed a significant
259 decrease in $\alpha 4\beta 7$ expression with a trend towards increased CCR7, potentially indicating
260 a reduction of NK cell trafficking to the gut, but instead mobilizing to the lymph nodes,
261 perhaps due to lymphoid follicles being reservoirs for both HIV and SIV(41). Despite the
262 effectiveness of cART, HIV can survive in follicular helper T cells(42-44), remaining
263 undetected by immunoregulatory activation. Using non-human primate models, previous
264 reports have compared the follicular regions in chronically infected SIV pathogenic and
265 non-pathogenic hosts and saw an increase of NK cell recruitment in elite
266 controllers(15,45). It is possible that in PWH, NK cells are mobilizing to the lymph nodes
267 in attempt to clear residual HIV infected T cells.

268 Another component of our study was to compare putative biomarkers for aging NK
269 cells in infected and uninfected individuals under 45 or over 50. Surprisingly, we found
270 that younger persons had NK cell repertoires that are relatively similar regardless of HIV
271 status, but noticeable disparities became clearly apparent in those over the age of 50.
272 CD2, CCR7, and $\alpha 4\beta 7$ were significant predictors compared to healthy donors for PWH
273 on cART, with the addition of CCR5 and CD85j for untreated PWH. Our data suggests
274 that neither aging nor HIV alone severely impacts the NK cell repertoire, but perhaps
275 rather a combination of the two does. Aging and HIV are associated with a variety of
276 similar cellular perturbations relating to function, proliferation, and exhaustion(23). A
277 comparison of elderly HIV-infected individuals to healthy donors in the same age cohort
278 found increased rates of hypertension, hypertriglyceridemia, and other disorders in HIV-
279 infected individuals(46). It is possible that a combination of the two have a synergistic and

280 significant effect on the NK cell phenotypic profile. Altogether, these data highlight the
281 age-associated NK cell phenotypic changes in HIV-infected adults and their potential
282 clinical implications.

283 In line with previous research, we identified NK cells from CMV infected aged adults
284 to have higher expression of CD57 and NKG2C(35,36,47), a unique subset of adaptive
285 NK cells primed by the virus. Although we found age-related NK cell phenotypic changes
286 also associated with CMV status, there was minimal impact of CMV infection specifically
287 on NK trafficking receptor expression in healthy donors. Specifically, there was no
288 significant difference between CMV+ and CMV- aged individuals for NK cell $\alpha4\beta7$, CCR7,
289 or CXCR6. These data suggest that age-related changes in the NK cell repertoire do
290 occur due to CMV infection but are largely independent from those induced by HIV.

291 Importantly, we acknowledge several limitations in our study which were
292 predominantly focused on limited treatment information and sample quality issues. Due
293 to the age or other limitations of some samples, we were unable to obtain CMV viral
294 shedding from cryopreserved urine samples. In addition, treatment history, specific
295 antiretroviral drugs used, and any potential interruptions in treatment were not known for
296 all participants. Additionally, as many study participants were on ART regimens that are
297 no longer common care, the impact of other drugs on NK cells and chronic inflammation
298 will need to be further examined.

299 Altogether, we provide an aging NK cell phenotypic profile, delineate the
300 modulations induced by HIV infection, and highlight the combined dysfunctional
301 properties of HIV and aging. To our knowledge, this is the first study of NK cell phenotypes
302 in aging PWH in both treated and untreated aged cohorts. NK cells have been linked to

303 the control and disease progression of HIV making it imperative to understand the natural
304 trafficking patterns of these cells and the subsequent modulations following HIV infection.
305 Further research will be needed to evaluate the functional capabilities of aged NK cells in
306 PWH on and off cART and the consequences of these changes.
307
308

309 **Materials and Methods**

310 **Flow cytometry**

311 Flow cytometry staining of PBMCs was performed using a 28-color surface phenotype
312 panel (Supplemental Table 2). Briefly, cryopreserved PBMCs were rapidly thawed at 37
313 °C and immediately transferred to complete media pre-warmed to 37 °C. After
314 centrifugation, PBMCs were incubated for 30 minutes at 4 °C in Blue Live/Dead
315 (Invitrogen, Carlsbad, CA). PBMCs were then washed and surface stained for 20 minutes
316 at room temperature. Samples were then stained immediately after with Streptavidin-
317 BUV395 as a secondary antibody for biotinylated CD159c for 15 minutes at RT. PBMCs
318 were washed once more and fixed with 2% paraformaldehyde. All acquisitions were
319 carried out on a FACS Symphony cytometer (BD Biosciences) and analyzed with FlowJo
320 v10.7.1 (BD Biosciences). The gating strategy used to define NK cells was as follows:
321 Live, Lineage^{-} (CD19^{-} CD14^{-} CD4^{-} CD3^{-}) CD56^{dim} CD16^{+}.

322

323 **CMV antibody titer quantification**

324 Matched cryopreserved plasma samples were used for quantification of Human
325 Cytomegalovirus IgG and IgM antibody titers (Quest Diagnostics; order code 6732;
326 Marlborough, MA, USA).

327

328 **HIV viral load quantification**

329 Plasma viral loads were assessed using Amplicor HIV-1 Monitor Ultrasensitive Assay
330 (Roche Molecular System, Branchburg, NJ).

331

332 **Multidimensional analyses**

333 Multidimensional scaling (MDS)(48) was performed using the CytoGLMM R package as
334 described by package documentation(49) using median summarized expression values
335 for each sample. Uniform Manifold Approximation and Projection (UMAP)(50) was
336 performed with a beta version of the CytoDRAV(51) application that implements the uwot
337 v0.1.10 R package(52). Input data were transformed using the inverse hyperbolic sine
338 function with a cofactor of 5. Default parameters were used and results visualized with
339 the ggplot2 R package(53).

340

341 **Statistical analysis**

342 Spearman correlation analysis was performed in Prism v9.0 (GraphPad Software).
343 Generalized linear modeling (GLM) with bootstrapping was performed with the
344 CytoGLMM R package(54) with R v3.6.3(55). GLM with bootstrapping was performed
345 according to previously published studies(56,57). Briefly, compensated FCS files of
346 $CD56^{dim}$ $CD16^{+}$ NK cells were exported from FlowJo and loaded into R. Data were
347 transformed using the hyperbolic sine transformation with a cofactor equal to 5 and a
348 random sampling of 2000 cells from each sample, or all cells if the sample contained
349 fewer than 2000 cells, was performed. The *cytoglm* function of the CytoGLMM package
350 was used to perform 1000 iterations of bootstrap resampling with replacement followed
351 by logistic regression. Results are reported as log-odds of a given marker predicting
352 binary group assignment (HD and ART; HD and PWH; HD under 45 and HD over 50).
353 Corrections for multiple testing were performed using the Benjamini-Hochberg method
354 for controlling false discovery rate(58). P-values reported are BH adjusted p-values. An

355 adjusted p-value cutoff of 0.05 was used to determine significance. Median fluorescence
356 intensities (MFI) were exported from FlowJo for comparing marker expression between
357 groups. These data were not transformed prior to statistical analysis. Mann-Whitney *U*-
358 tests were used to compare MFI levels between groups.
359
360 **Study approval**
361 Cryopreserved human peripheral blood mononuclear cells (PBMCs) were obtained from
362 the Hawai'i Aging with HIV-1 Cohort (HAHC) at University of Hawaii. Details of the HAHC
363 study enrollment and clinical characterization were previously published(59) and
364 approved by the University of Hawai'i Institutional Review Board. All participants signed
365 institutional review board–approved informed consent forms prior to participation. CD4
366 lymphocyte counts were obtained in real-time by standard technique from a local CAP
367 certified reference laboratory. Demographic and clinical details of study participants are
368 provided in Supplemental Table 1.
369

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Conflicts of interest

All authors report no financial conflicts of interest.

Author Contributions

L.C.N. and R.K.R. designed the study. K.W.K., S.V.S., O.A.L., T.A.P., M.J.C., M.M., and S.B. performed the experiments and analyzed data. K.W.K. performed the bioinformatic analyses. C.M.S. provided samples critical to the study. All authors contributed to the writing of the manuscript.

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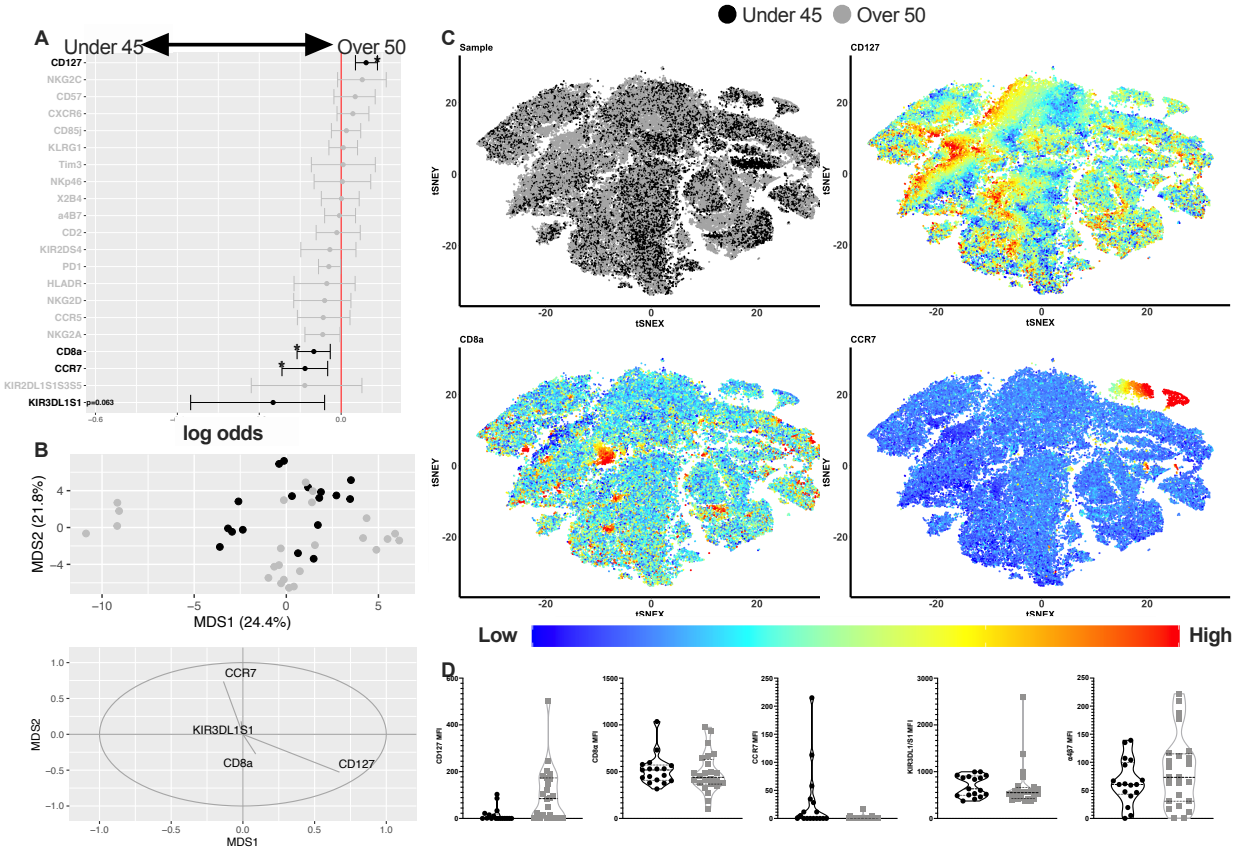

Figure 1. Analysis of aging on CD56^{dim} CD16^{+} NK cells. (A) Generalized linear model (GLM) with bootstrap resampling was performed to determine significant predictors of age group in HD. (B) Multidimensional scaling (MDS) was performed to determine clustering in dimensionality reduced projection. Black circles represent HD under the age of 45 and grey circle represent HD over the age of 50. MDS loadings are displayed for the significant predictors from A. (C) t-Distributed Stochastic Neighbor Embedding (t-SNE) was performed using sampling approach from A. Relative marker expression overlays use colorimetric scale from low (blue) to high (red) relative expression of the protein. (D) Violin plots of median fluorescence intensity (MFI) for significant predictors of age group. *BH adjusted $p < 0.05$

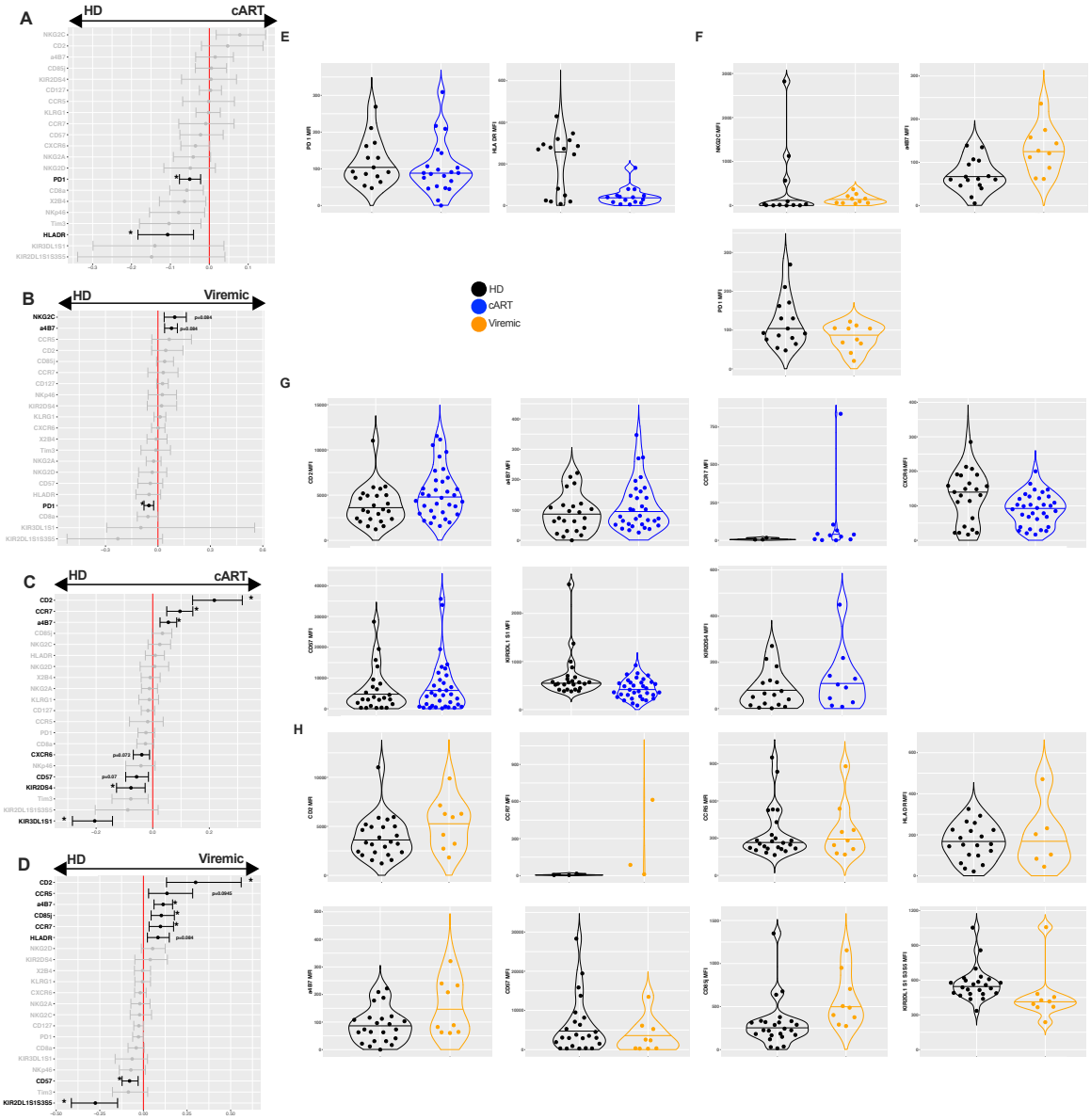

Figure 2. GLM with bootstrap resampling was used to determine predictors of (A) HD and cART under the age of 45; (B) HD and Viremic PWH under the age of 45; (C) HD and cART over the age of 50; and (D) HD and Viremic PWH over the age of 50. Violin plots show expression levels of highlighted markers. (E) HD and cART under 45. (F) HD and Viremic PWH under 45. (G) HD and cART over 50. (H) HD and Viremic PWH over 50. Black circles represent HD, blue circles represent cART, and orange circles represent Viremic PWH. *BH adjusted $p < 0.05$

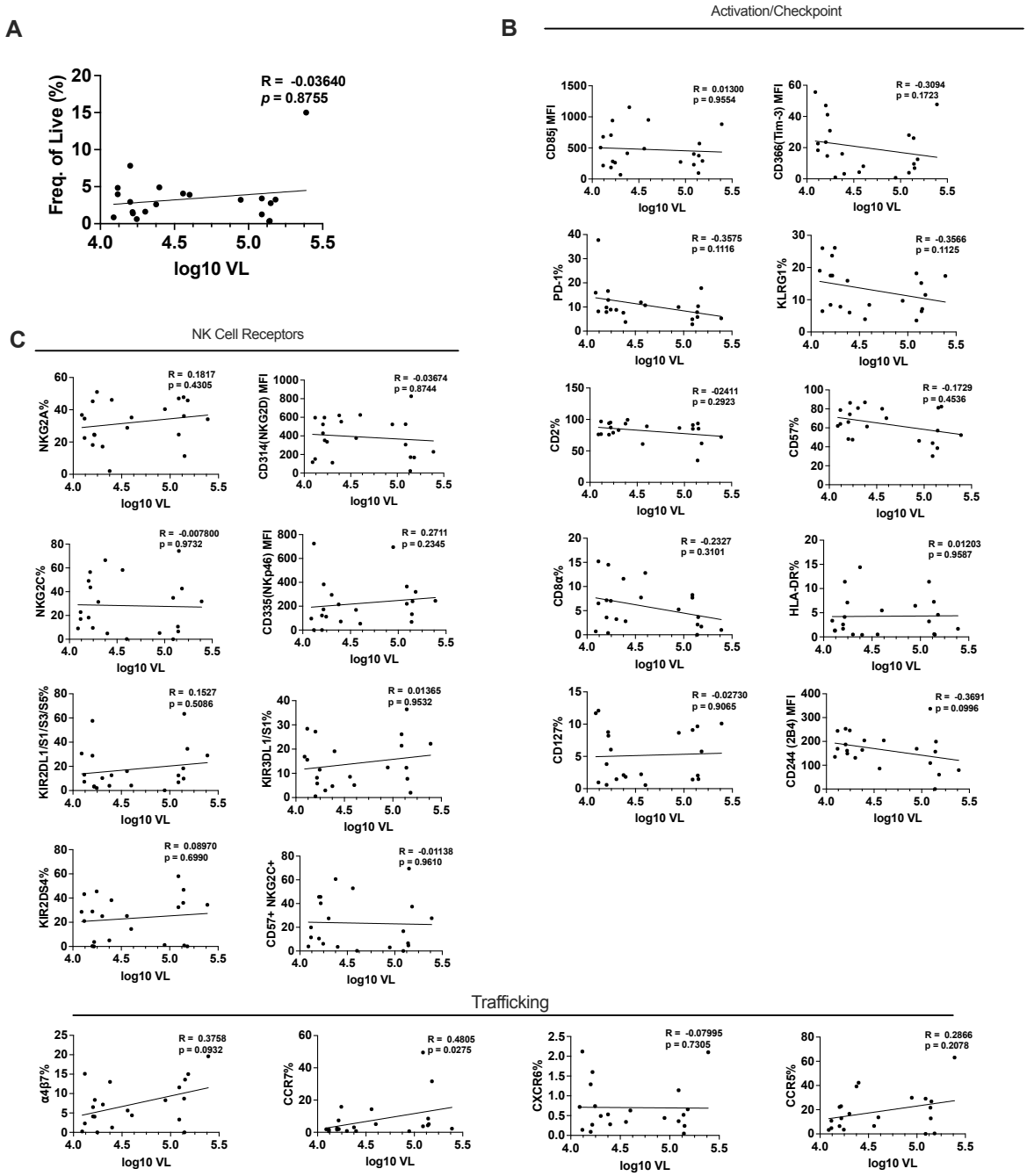

Figure 3. Correlation analysis of HIV viral load and protein expression levels on CD56^{dim} CD16^{+} NK cells in PWH. (A) CD56^{dim} CD16^{+} NK cell frequency of live lymphocytes. (B) Activation and/or checkpoint receptors. (C) NK cell receptors including NCRs and NKG2 family receptors. (D) Trafficking receptors including mucosae-homing marker α4β7 and lymph-node homing marker CCR7. Spearman R and *p*-values noted on each graph.

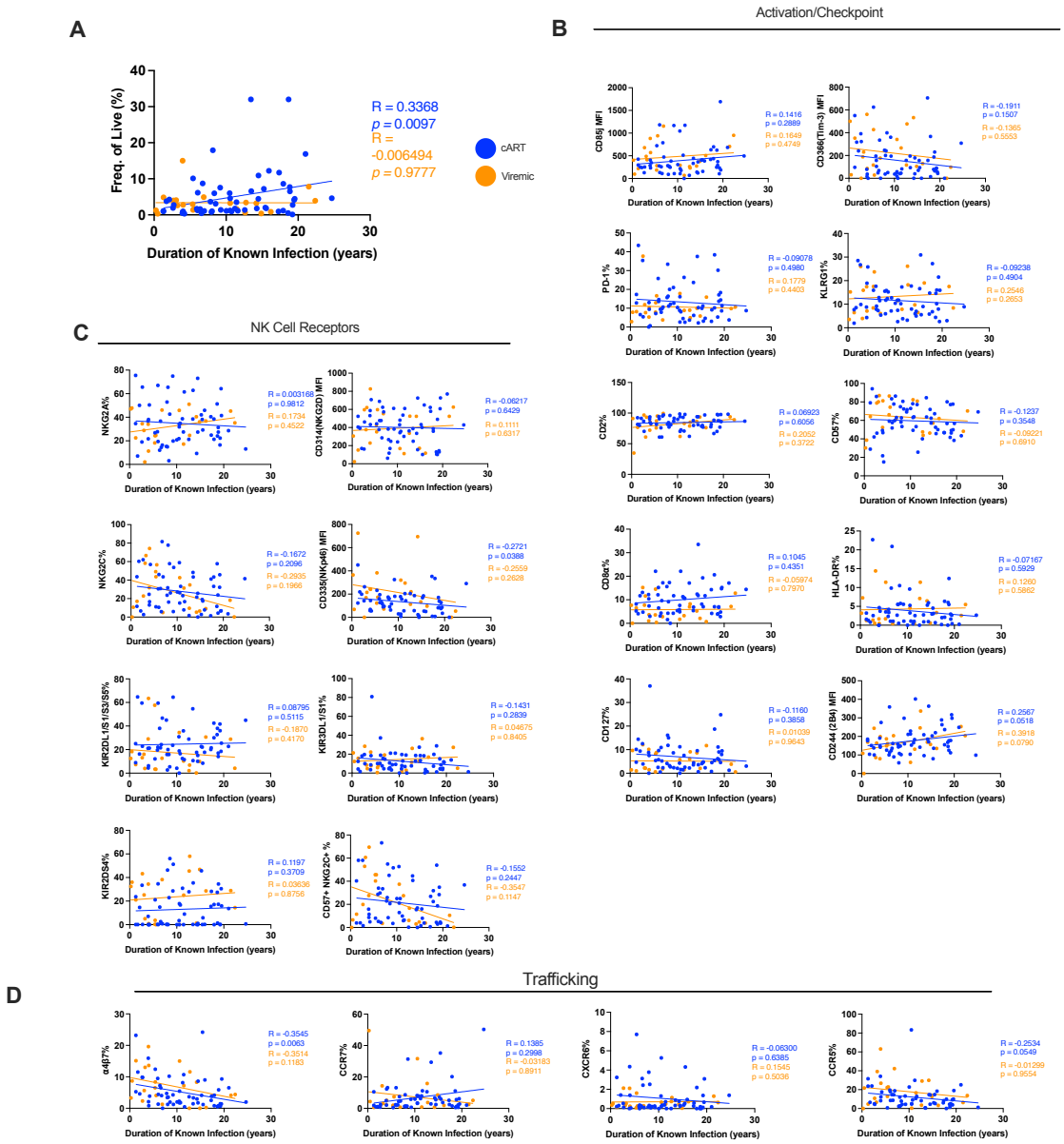

Figure 4. Correlation analysis of duration of known infection in PWH and NK protein expression. (A) CD56^{dim} CD16^{+} NK cell frequency of live lymphocytes. (B) Activation and/or checkpoint receptors. (C) NK cell receptors including NCRs and NKG2 family receptors. (D) Trafficking receptors including mucosae-homing marker α4β7 and lymph-node homing marker CCR7. Blue circles, lines, and text represent results of cART. Orange circles, lines, and text represent results of Viremic PWH. Spearman correlation was used for determining correlation and significance.

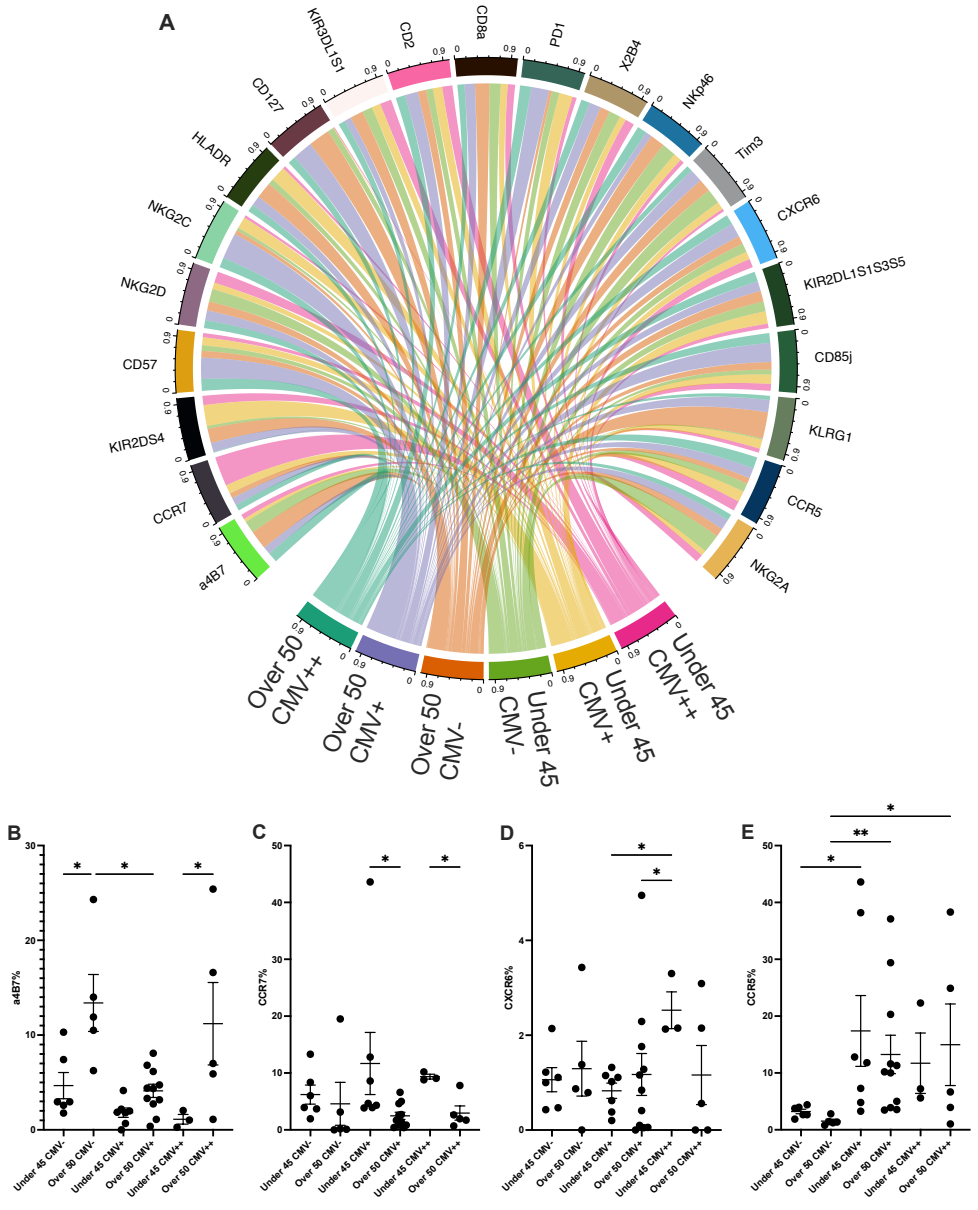

Figure 5. Analysis of NK protein expression based on age and CMV status. (A) Chord plot showing proportion of median fluorescence intensity for all markers in HD, delineated by age group and CMV titer. Expression levels of trafficking markers in HD based on CMV titer comparing the expression of α4β7 (B), CCR7 (C), CXCR6 (D), and CCR5 (E). * $p < 0.05$; ** $p < 0.01$. Kruskal-Wallis test followed by Benjamini Hochberg FDR correction for multiple testing.